Chemistry & Biology
Review

Small Molecules for Dissecting Endomembrane Trafficking: A Cross-Systems View

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http://dx.doi.org/10.1016/j.chembiol.2013.03.009

Endomembrane trafficking has a key role for ensuring homeostasis, growth and development, hormonal signaling, and adaptation of eukaryotes to the constantly changing environmental conditions. The complex organization of the endomembrane system implies the need for searching novel tools to specifically probe the regulatory components and dissect the tightly interconnected vesicle transport pathways. Here, we review the large-scale chemical genetic screens, which led to the identification of small molecules with an impact on various parts of the vesicle trafficking network. We discuss the similarities and differences in the organization of the endomembrane systems in yeasts, mammals, and plants based on studies of small molecules and their effects on trafficking hubs, routes, and conserved protein targets.

Eukaryotic cells are characterized by functional compartmentalization of macromolecule trafficking, which is performed by an elaborate and fine-tuned endomembrane system. The endomembrane network comprises dynamic organelles with discrete morphology, localization, and functions and ensures both the secretion of biomolecules and the uptake of extracellular material that is delivered to intracellular locations in a highly coordinated manner. The exchange of cargo, membrane components, and solutes between the cellular compartments is a continuously ongoing process that involves alternating steps of membrane deformation, budding, fission, tethering, and fusion. The secretory and endocytic pathways are largely interconnected at the level of common trafficking hubs and protein regulators, which substantially increases the complexity of the endomembrane system. When key protein components of the endomembrane system are compromised, pleiotropic phenotypes may occur, making it difficult to elucidate the underlying molecular mechanism with classical genetic approaches. Significant limitations of forward and reverse genetics are the lack of phenotypic changes if functionally redundant components of vesicular trafficking are analyzed and, in the opposite case, the lethality of loss-of-function mutants due to the essential role of the intracellular transport for cell viability (Hicks and Raikhel, 2012). An alternative approach that circumvents these limitations deals with the use of small organic molecules as specific effectors of endomembrane trafficking. An important advantage of chemical genetics in dissecting complex biological processes is the enormous diversity of chemical structures that could be used for probing protein functions in a dose-dependent and reversible manner (Kaschani and van der Hoorn, 2007). Hence, it should be possible to perturb almost any protein in a specific way, allowing the targeting of essential genes. In addition, entire gene families can be targeted by bioactive chemicals through perturbation of a common feature of the corresponding proteins, thus addressing redundancy among genes (Hicks and Raikhel, 2012; Tóth and van der Hoorn, 2010).

Recent advances in systems biology, bioinformatics, and modern cell imaging opened novel opportunities for identifying small molecules that can be used as tools to dissect endomembrane trafficking pathways. Here, we outline the progress in current chemical genetics related to intracellular vesicle trafficking, with an emphasis on the use of small molecules in plants, mammals, and yeasts identified through high-throughput screens (Figure 1). The central routes and hubs of the endomembrane trafficking network in eukaryotic cells will be discussed for their sensitivity to the compounds (Figure 2; Table 1), which is determined by the extent of conservation of the protein targets.

Endomembrane Trafficking Routes in Yeasts, Plants, and Mammals: A Comparative View

The eukaryotic endomembrane system is composed of membrane-delimited organelles, small vesicular compartments that shuttle between those organelles, and a spectrum of transiently associated, peripheral membrane components from the cytosol that support, regulate, and define endomembrane trafficking (Foresti and Denecke, 2008). Proteins that are cotranslationally translocated into the endoplasmic reticulum (ER) are subsequently sorted for trafficking through the Golgi apparatus and the *trans*-Golgi network (TGN). Anterograde transport from the ER is mediated by COPII-coated vesicles, which fuse with pre-Golgi intermediates to release their cargo, while Golgi-to-ER protein recycling requires COPI vesicle formation. Unlike mammals, the plant endomembrane system lacks an ER-Golgi intermediate compartment (Foresti and Denecke, 2008). After reaching the TGN, secretory proteins are packaged into secretory vesicles and targeted to the plasma membrane (PM) (Vázquez-Martínez et al., 2012). So far, the secretory route from the TGN to the PM in plants is poorly understood. The TGN compartment also produces clathrin-coated vesicles directed to the lytic compartment (the lysosome in mammalian cells or the vacuole in plants and yeasts). The latter route involves late endosomes (LEs) in mammals, also called prevacuolar compartments/multivesicular bodies (PVCs/MVBs) in plant cells. Unlike mammals,

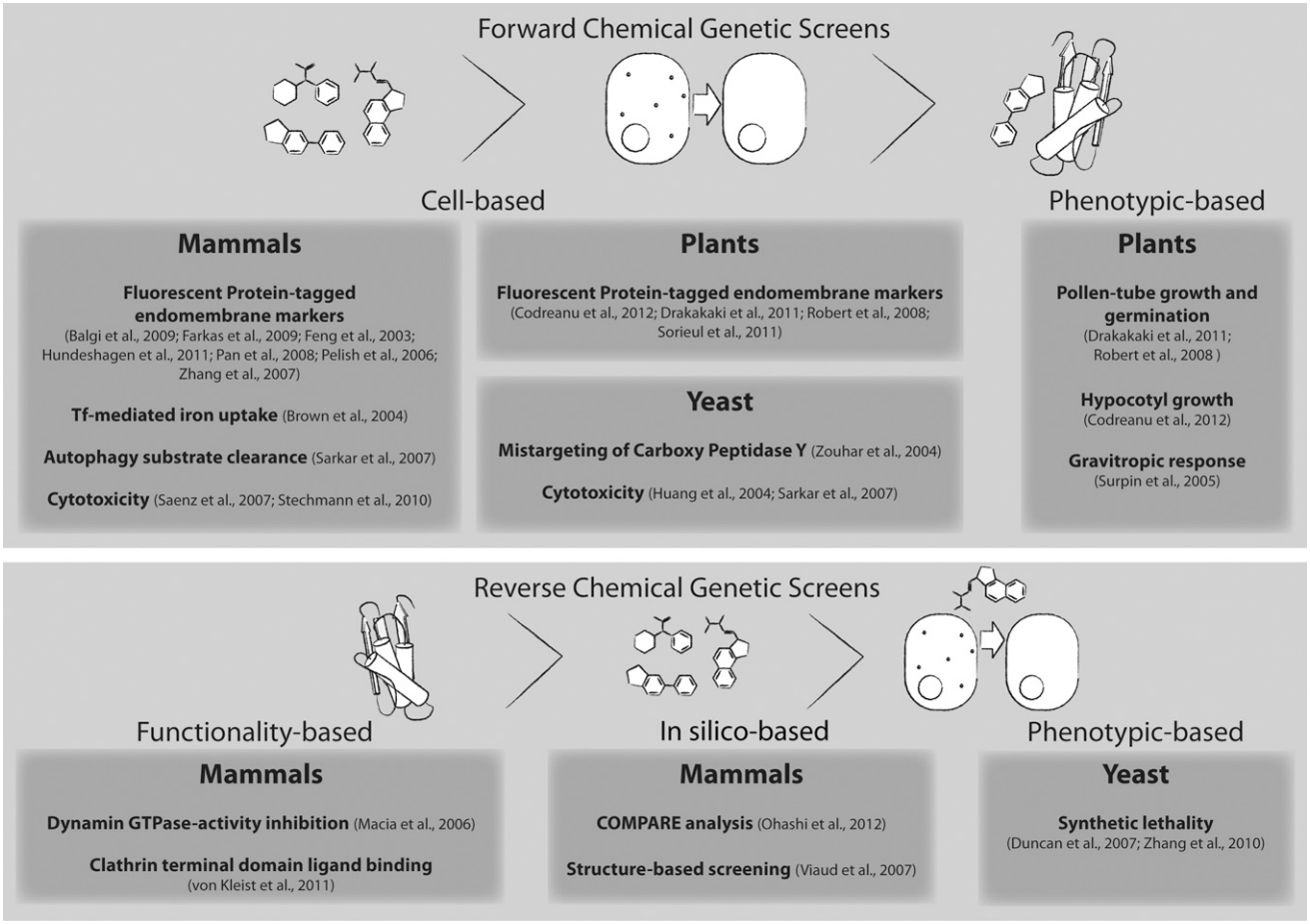


Figure 1. Forward and Reverse Chemical Genetic Screens across Yeasts, Plants, and Mammals
Forward chemical genetics is represented by cell-based and phenotypic-based screens. Reverse chemical genetics is represented by functionality-based, in silico-based, and phenotypic-based screens. Only the relevant systems are shown for each screen.

the post-TGN trafficking to the lytic vacuole in plants appears to occur independently of clathrin (Scheuring et al., 2011). Trafficking routes bypassing the LE/PVC/MVB compartment have also been reported in yeasts, mammals, and plants (Dell'Angelica, 2009; Feraru et al., 2010).

The endocytic pathway begins with invagination of the PM and formation of clathrin-coated vesicles through membrane fission. Similar to mammalian and yeast cells, clathrin-mediated endocytosis (CME) appears to be an essential trafficking route in plants, indicating the conservation throughout evolution (Chen et al., 2011). Besides CME, growing evidence supports the importance of clathrin-independent endocytosis in mammals, yeasts, and plants (Howes et al., 2010; Li et al., 2012). Most of the cargo internalized by endocytosis undergoes recycling back to the PM. In contrast to mammals, the mechanisms of PM protein recycling in plants are still obscure. A small fraction of the endocytosed cargo is targeted for degradation to the lysosome by passing through early endosomes (EEs), which will gradually maturate into LEs (Huotari and Helenius, 2011). Early endosomes are subjected to gradual maturation to LEs (Huotari and Helenius, 2011). Recently, the maturation-based sorting model known from mammalian systems has been considered in plants in which MVBs/LEs are most likely formed through gradual transformation of parts of the TGN (Scheuring et al., 2011).

An additional route to the lysosome and vacuole is provided by autophagy. This process involves the enclosure of cellular contents, such as long-lived proteins and organelles by a double membrane structure to form vesicles, called autophagosomes, which will eventually fuse with the lysosome or vacuole in order to degrade their content. Autophagy is an essential process for recycling cellular contents in yeasts, plants, and mammals (Li and Vierstra, 2012; Yang and Klionsky, 2010). The identification of autophagy-related (*ATG*) genes in *Saccharomyces cerevisiae* and subsequently in other organisms revealed that the molecular machinery responsible for autophagy seems to be well conserved, although plants appear to have a much more diverse array of *ATG* genes at their disposal (Li and Vierstra, 2012).

Although the fundamental mechanism of intracellular vesicle trafficking appears to be conserved in eukaryotes, there are several notable differences. In contrast to mammalian cells, the TGN in plants is morphologically and functionally distinguished from the *trans* Golgi cisternae (Viotti et al., 2010). A number of experimental evidences revealed that the plant TGN possesses

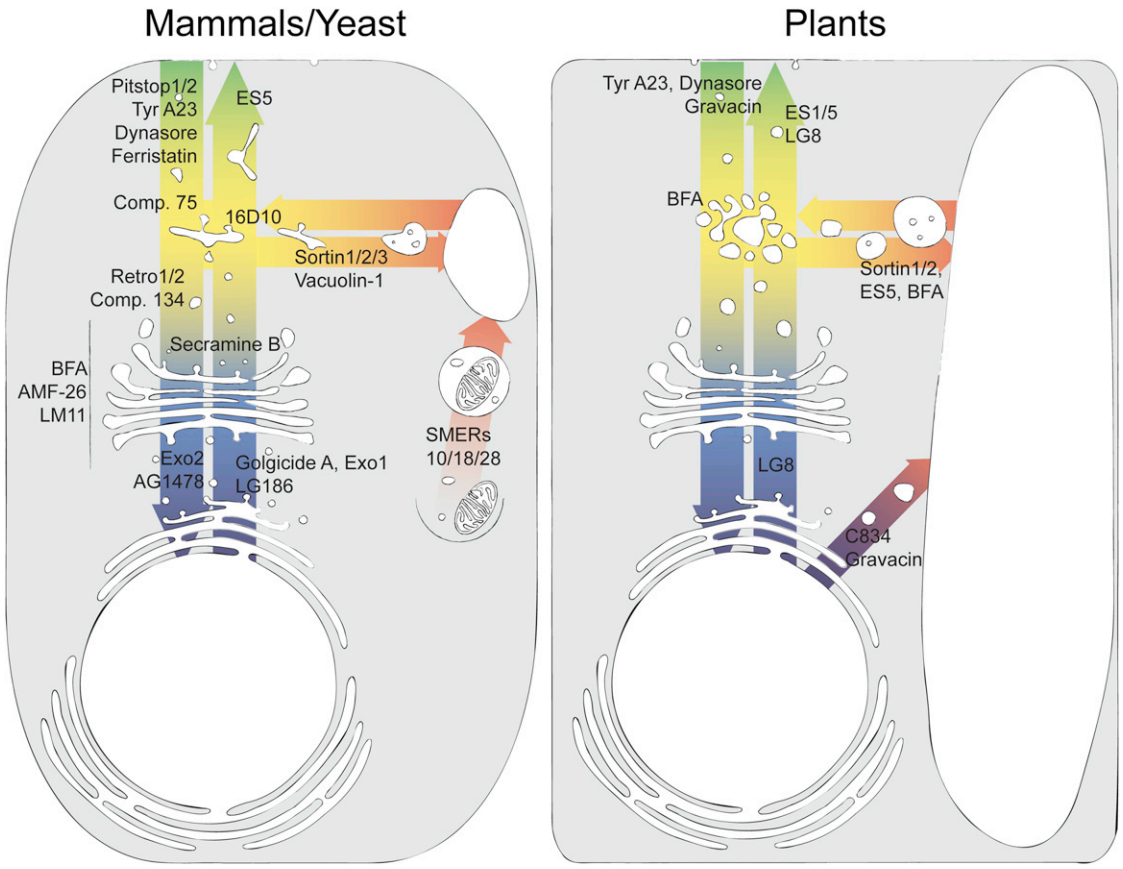


Figure 2. Schematic Representation of Major Trafficking Routes and Their Small-Molecule Effectors in Different Systems
Shown are a mammalian/yeast cell (left) and plant cell (right). Only endomembrane compartments relevant for this review are shown. Discussed small molecules are indicated close to the compartment or route they affect. Dark blue, nucleus/ER; light blue, Golgi; yellow, EE (mammalian/yeast) or TGN (plants). Yellow to green route, exocytic or recycling pathway with recycling endosomes; green to yellow route, endocytic pathway with endocytic vesicles maturating into EEs. Yellow to red (lysosome/vacuole) route, LE and/or MVB maturation from EE into lysosome/vacuole. Dark blue to red route, Golgi/TGN-independent trafficking from ER to vacuole. Gray (cytosol) to red route, autophagy. Note the absence of an autophagic route in the plant cell, since rapamycin has not been shown to affect autophagy in plant cells yet.

features of EEs and is involved in both endocytic and secretory pathways. According to the latest model, the TGN in plants is subdivided into domains where secretory vesicles are released to the PM, into domains releasing clathrin-coated vesicles for recycling to the PM and into domains that mature into MVBs (Scheuring et al., 2011). Another specific feature of the yeast and plant endomembrane systems is the vacuole, which in the plant cell occupies up to 90% of the total cell volume (Surpin and Raikhel, 2004). Two types of functionally distinct vacuoles coexist in the plant cell, namely protein storage vacuoles (PSVs) and lytic vacuoles, which are analogous to lysosomes in mammalian cells (Zouhar and Rojo, 2009). The PSVs can be distinguished by the presence of reserve proteins used mainly during seed germination and by their neutral pH in contrast to lytic vacuoles. However, the two types of plant vacuoles can fuse and give rise to a large central vacuole (Frigerio et al., 2008).

In Search for Small-Molecule Modifiers of Endomembrane Trafficking

Small bioactive molecules have long been used in medicine and agriculture (Cong et al., 2012; Delaney et al., 2006). Recently, large-scale systematic screens have boosted the search for novel chemical regulators for mammalian drug discovery, and this strategy is being rapidly adopted in plants (Hicks and Raikhel, 2012; Knight and Shokat, 2007). Two types of chemical genetics screens (forward and reverse) have been employed to dissect the endomembrane trafficking in eukaryotes (Figure 1).

Forward chemical genetics uses growth phenotype- or cell-based screens for inhibition or activation of a particular cellular process, followed by molecular target identification. Growth phenotype-based forward chemical genetics screens for endomembrane trafficking modulators have been recently carried out in plants where a high-throughput screen based on the germination and growth of tobacco pollen was used to interrogate more than 46,000 compounds (Drakakaki et al., 2011; Robert et al., 2008). Other growth phenotypes related to endomembrane systems, such as gravitropic responses or cell elongation during hypocotyl growth, were also used in chemical genetics screens (Codreanu et al., 2012; Surpin et al., 2005).

The cell-based screens often employ the use of fluorescent marker proteins as readouts for activity or inhibition of trafficking pathways. Examples from the mammalian systems are the

Table 1. Small Molecule Effectors of Endomembrane Trafficking Identified through Large-Scale Chemical Genetic Screens

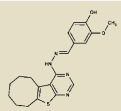
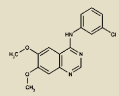
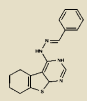
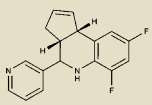
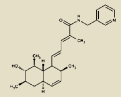
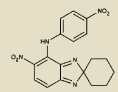
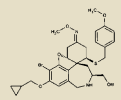
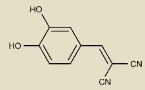
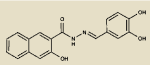
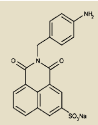
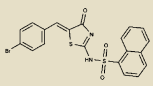
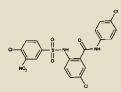
Name	Structure	Affected Endomembrane Compartment	Known Target	Screen	References
Chemical Effectors of Exocytosis/Secretion/Anterograde Trafficking					
Exo1		ER to Golgi trafficking		fluorescence-based imaging screen of VSV-G^{ts} trafficking	Feng et al., 2003
Exo2		ER to Golgi trafficking, Golgi, TGN	Arf-GEF GBF1	fluorescence-based imaging screen of VSV-G^{ts} trafficking	Spooner et al., 2008
LG186		Golgi	Arf-GEF GBF1		Boal et al., 2010
AG1478		*cis*-Golgi		P58-YFP-based screen in H4 human glioblastoma cells	Pan et al., 2008
LG8		ER, Golgi		Exo2 analog screen for activity in *A. thaliana*	Sorieul et al., 2011
Golgicide A		Golgi	Arf-GEF GBF1	luciferase-based toxin susceptibility screen	Sáenz et al., 2009
AMF-26		*cis*- and *trans*-Golgi		COMPARE-guided in silico screening	Ohashi et al., 2012
LM11		Golgi, endosomal structures	Arf1-GDP/ARNO complex	in silico structure-based screen	Viaud et al., 2007
Secramine B		Golgi to PM trafficking	Rho GTPase Cdc42	fluorescence-based imaging screen of VSV-G^{ts} trafficking	Pelish et al., 2006
Chemical Effectors of Endocytosis/Retrograde Trafficking					
Tyrphostin A23		endocytosis	interfering between tyrosine motifs and AP2 complex	screen for tyrosine kinases	Yaish et al., 1988
Dynasore		endocytosis	dynamin	dynamin GTPase activity inhibitor screen	Macia et al., 2006
Pitstop 1		endocytosis	clathrin terminal domain (TD)	ELISA-based screen for inhibition of amphiphysin and clathrin TD association	von Kleist et al., 2011
Pitstop 2		endocytosis	clathrin TD	ELISA-based screen for inhibition of amphiphysin and clathrin TD association	von Kleist et al., 2011
16D10		endosomes, lysosomes	likely inhibition of V-ATPases or as proton ionophore	fluorescence-based imaging screen of VSV-G^{ts} trafficking	Nieland et al., 2004

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Table 1. ***Continued***

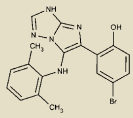
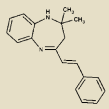
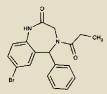
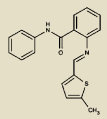
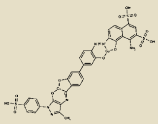
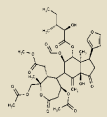
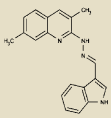
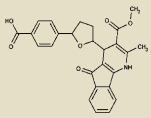
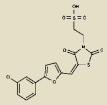
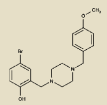
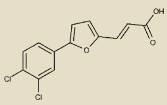
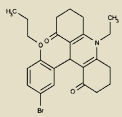
Name	Structure	Affected Endomembrane Compartment	Known Target	Screen	References
Comp. 75		early retrograde transport		luciferase-based toxin susceptibility screen	Saenz et al., 2007
Comp. 134		recycling endosomes to Golgi, retrograde transport		luciferase-based toxin susceptibility screen	Saenz et al., 2007
Retro-1		EE to TGN retrograde trafficking		screen for inhibitors of ricin toxicity	Stechmann et al., 2010
Retro-2		EE to TGN retrograde trafficking		screen for inhibitors of ricin toxicity	Stechmann et al., 2010
Ferristatin		increased transferrin receptor internalization and degradation		fluorescence-based screen for calcein quenching	Brown et al., 2004
ES1		endosomes		screen for pollen tube growth and germination inhibitors	Robert et al., 2008
ES5		endosomes, recycling		screen for pollen tube growth and germination inhibitors	Drakakaki et al., 2011
Chemical Effectors of Vacuolar Trafficking and Autophagy					
Sortin 1		vacuolar traffic and morphology		screen for mistargeting of carboxypeptidase Y	Zouhar et al., 2004
Sortin 2		vacuolar traffic and morphology		screen for mistargeting of carboxypeptidase Y	Zouhar et al., 2004
Sortin 3		vacuolar traffic and morphology		screen for mistargeting of carboxypeptidase Y	Zouhar et al., 2004
Gravacin		ER to vacuole trafficking	PGP19	screen for gravitropic response	Surpin et al., 2005
C834		vacuolar trafficking		screen for pollen tube growth and germination inhibitors	Rivera-Serrano et al., 2012

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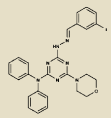
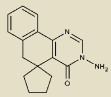
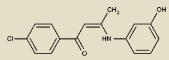
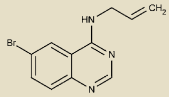
Table 1. *Continued*

Name	Structure	Affected Endomembrane Compartment	Known Target	Screen	References
Vacuolin-1		induction of vacuole formation, inhibition of exocytic PM-lysosome fusion		fluorescence-based imaging screen of VSV-G^{ts} trafficking	Cerny et al., 2004
SMER 10		enhancer of autophagy		cytotoxicity screen in yeast and autophagy substrate clearance in mammals	Sarkar et al., 2007
SMER 18		enhancer of autophagy		cytotoxicity screen in yeast and autophagy substrate clearance in mammals	Sarkar et al., 2007
SMER 28		enhancer of autophagy		cytotoxicity screen in yeast and autophagy substrate clearance in mammals	Sarkar et al., 2007

high-throughput screen for inhibitors of exocytosis using BSC1 fibroblast cells infected with GFP-fused vesicular stomatitis virus (VSVG^{ts}-GFP) (Feng et al., 2003), the screen for inhibitors of endocytosis based on transferrin (Tf) receptor-mediated iron uptake in HeLa cells measured by the quenching of calcein fluorescence (Brown et al., 2004), and the screen for inhibitors of Golgi-mediated trafficking based on the expression of the yellow fluorescent protein (YFP)-tagged Golgi-residing protein p58 (Pan et al., 2008). Several screens based on autophagosome markers coupled with either GFP or luciferase have assessed the ability of small molecules to affect autophagy (Balgi et al., 2009; Farkas et al., 2009, 2011; Zhang et al., 2007). Flow cytometry has also been used to quantify fluorescent protein-based sensors for autophagic and endolysosomal activities in a screen for autophagy modulators (Hundeshagen et al., 2011). In plants, PM proteins or other endomembrane compartment markers tagged with fluorescent proteins have been used as markers to identify compounds affecting endocytosis, recycling, or vacuolar morphogenesis (Codreanu et al., 2012; Drakakaki et al., 2011; Robert et al., 2008; Surpin et al., 2005).

Cell-based screens for endomembrane trafficking modulators can also employ other readouts, such as cell viability, enzymatic reactions, or secretion. For example, cytotoxicity measurements using destabilized firefly luciferase (Saenz et al., 2007) or radioactive-labeled amino acids (Stechmann et al., 2010) were used in screens for inhibitors of retrograde trafficking of toxins (shiga, cholera, and ricin) in mammalian cells. Small-molecule enhancers and inhibitors of autophagy were identified in a screen for their ability to affect the clearance of the autophagy substrate A53T α-synuclein in mammalian cells (Huang et al., 2004; Sarkar et al., 2007). In yeasts, high-throughput immunoassays for detection of missecreted carboxypeptidase Y have been performed to screen for bioactive compounds that interfere with the sorting of lytic enzymes to the vacuolar lumen (Zouhar et al., 2004).

Reverse chemical genetics searches for compounds that inhibit or activate in vitro proteins known to be involved in a definite process followed by further characterization of the phenotypic effects at cellular and/or organism level (Figure 1). In comparison to forward chemical genetics, this approach has the significant advantage of bypassing the target identification step. This strategy has been successful in identifying chemical modulators of protein trafficking components involved in early steps of CME, such as Dynasore and Pitstops (Macia et al., 2006; von Kleist et al., 2011).

Because of the available large collections of deletion mutants, the yeast *S. cerevisiae* has become a powerful tool for small molecule discovery by using synthetic lethality (reviewed by Roemer et al., 2012). Synthetic lethality screens were used to identify inhibitors of exocytosis (Zhang et al., 2010) and the trafficking between the TGN and the endosomes (Duncan et al., 2007). Whole-genome yeast deletion collections have also been screened for responses to chemical libraries in diverse environmental conditions, where genes involved in endomembrane trafficking and vacuolar degradation were found essential for resistance to multiple compounds (Hillenmeyer et al., 2008).

Alternative in silico screening approaches have enabled the search for novel exocytosis inhibitors in mammals. A virtual screen has been designed to specifically probe the interaction between the ADP-ribosylation factor 1 (ARF1) guanosine triphosphatase (GTPase) and its guanine exchange factor (GEF) ARNO based on known crystal structures of their complex (Viaud et al., 2007). Drug sensitivity databases have also been exploited to find novel inhibitors of ARF1 GTPase activation based on functional correlation with compounds with already known properties (Ohashi et al., 2012).

Chemical Effectors of Exocytosis/Secretion/Anterograde Trafficking

In the early 2000s, only very few small molecule modulators of the secretory pathways were known. The best example of such an inhibitor of secretion in mammalian cells is the fungal macrocyclic lactone Brefeldin A (BFA), which has been instrumental for studying the function of the Golgi

(Lippincott-Schwartz et al., 1991). Structural insights into the mechanism of BFA action in mammalian cells have revealed that this compound impedes the interaction between different members of the ARF GTPase family and their associated large GEFs and, thus, causes a diversity of effects, including tubulation of EEs, redistribution and fusion of the TGN with EEs, and fusion of the ER with the Golgi (Dinter and Berger, 1998; Renault et al., 2003). In contrast to mammalian and yeast systems, plants, for example, *Arabidopsis,* have only large ARF-GEFs, of which some are resistant to BFA (Anders and Jürgens, 2008). In plants, BFA has been described as an inhibitor of either secretory or endocytic pathways, depending on where in the cell the BFA-sensitive ARF-GEFs are residing (Nebenführ et al., 2002; Robinson et al., 2008). Chemical genetics has been used to search for small molecules with more specific effects on exocytosis than BFA in mammalian systems (Feng et al., 2003; Figure 2; Table 1). Two compounds structurally different from BFA, Exo1 and Exo2, were found to inhibit the traffic from the ER to the Golgi. Although the direct targets of Exo1 and Exo2 are not known, their mode of action differs from that of BFA (Feng et al., 2003, 2004). Treatment with either Exo1 or Exo2 induces reversible redistribution of Golgi material back to the ER without any concomitant effects on endocytic pathways. In contrast to BFA, Exo1 does not directly inhibit ARF-GEFs and has less effect on the organization of the TGN (Feng et al., 2003). The effect of Exo2 on the Golgi and the TGN is similar to that of BFA, except that it does not induce tubulation and merging of the TGN and endosomal compartments (Feng et al., 2004; Spooner et al., 2008). Therefore, it was proposed that Exo2 is more selective than BFA, possibly inhibiting the function of only the *cis*-Golgi-residing ARF-GEF, GBF1. A modified version of Exo2, LG186, was further developed in order to increase selectivity toward GBF1 (Boal et al., 2010). Another chemical modifier of GBF1 activity, AG1478, was also found to preferentially target the *cis*-Golgi in human cell lines (Pan et al., 2008).

In contrast to mammalian cells, Exo2 was not active in plant cells (Sorieul et al., 2011). Screening a number of Exo2 derivatives revealed LG8 as an active analog in *Arabidopsis* root cells that causes severe changes in the morphology of the ER and the Golgi but not the post-Golgi compartments. Unlike BFA, the intracellular targets of LG8 in plants still remain elusive (Sorieul et al., 2011).

A high-throughput screen for small molecules that inhibit the toxin retrograde transport has identified an inhibitor of protein secretion called Golgicide A (GCA) (Saenz et al., 2007, Sáenz et al., 2009). GCA exhibits BFA- and LG186-like effects by specifically targeting GBF1 (Boal et al., 2010; Sáenz et al., 2009). Other recently identified exocytosis inhibitors, such as AMF-26 and LM11, have been designed to specifically impair the interaction between the ARF1 GTPase and its GEFs in mammals. Although the chemical structure of AMF-26 differs from that of BFA, this compound induces disruption of the *cis*- and *trans*-Golgi, targeting the activation of ARF1 GTPase (Ohashi et al., 2012). To date, BFA is known to inhibit only large but not small- and medium-sized ARF-GEFs (Anders and Jürgens, 2008). Conversely, LM11 targets the interaction between ARF1 and ARNO, a BFA-insensitive human ARF-GEF. This compound has been shown to induce changes in the Golgi similar to those provoked by BFA; however, it leads to the formation of large endosomal structures differing from the phenotype observed after BFA treatment (Viaud et al., 2007).

The mechanisms of regulation of the anterograde transport from the Golgi to the PM have been approached with the help of Secramine (Pelish et al., 2006). In mammalian cells, this compound has been shown to perturb the protein export from the Golgi by inhibiting the activation of Cdc42, revealing the essential role of this Rho GTPase for vesicle trafficking. In particular, Secramine targets the association of Cdc42 with its guanine dissociation inhibitor RhoGDI1 and prevents binding of this Rho GTPase to the Golgi membranes.

Chemical Effectors of Endocytosis/Retrograde Trafficking

A common way to interfere with endocytosis in mammals and plants (Irani and Russinova, 2009; Robinson et al., 2008) is the use of Tyrphostin A23, a low molecular weight compound, which has originally been identified in a chemical screen for inhibitors of protein tyrosine kinases (Yaish et al., 1988). It has been found that Tyrphostin A23 perturbs the interaction between the AP-2 adaptor complex and consensus tyrosine-containing motifs in the cytosolic domain of PM cargoes, leading to a block of cargo-sorting in clathrin-coated vesicles (Banbury et al., 2003). Tyrphostin A23 inhibits both endocytosis and vesiculation at the TGN in plants (Robinson et al., 2008). Although the function of the plant AP-2 complex is most likely conserved in plants, the target of Tyrphostin A23 has not yet been defined. Nevertheless, the use of this compound has been instrumental in studies of CME in plants (Irani et al., 2012; Ortiz-Zapater et al., 2006).

CME in mammals has been successfully approached by means of reverse chemical genetics (Figure 2; Table 1), which has been facilitated by the considerable knowledge about the CME machinery in this system. Dynasore has been identified as a noncompetitive and reversible inhibitor of the GTPase activity of dynamin, a protein essential for vesicle formation in CME and caveolae-mediated trafficking (Macia et al., 2006). Dynasore arrests the progression of vesicle coat assembly and the subsequent uncoating. Most of the plant genomes encode dynamin-related proteins, some of which might function in CME (Fujimoto and Ueda, 2012). A recent study on the intracellular trafficking of the fungal protein elicitor receptor, LeEix2, demonstrated the use of Dynasore for blocking endocytosis in plant cells (Sharfman et al., 2011).

Two structurally different compounds, Pitstop 1 and Pitstop 2, have also been used to elucidate the mechanisms of clathrin-coated pit formation in mammals (von Kleist et al., 2011). Pitstops have been found to compete with clathrin box-containing accessory proteins for a common binding site on the terminal domain of the clathrin heavy chain. Interference with the function of this domain does not affect clathrin recruitment, but Pitstops severely perturb clathrin-coated pit dynamics, due to the inability of clathrin to bind regulatory proteins required for progression in vesicle maturation. Similar to Dynasore, Pitstops induce the accumulation of endocytic intermediates at the PM, representing different stages of this trafficking route. Pitstop 2 was shown to be effective in blocking the internalization of PM cargo markers, like the Tf receptor and the epidermal growth factor in mammalian cells, through selective impairment of CME (von Kleist et al., 2011). A recent report has indicated that, in addition to CME,

Pitstop 2 is a potent inhibitor of protein internalization via clathrin-independent endocytic pathways (Dutta et al., 2012).

Several compounds identified through forward chemical genetics have been found to target endosomal compartments from the endocytic pathway downstream of the PM. A group of sulfonamides (with a representative member, the compound 16D10) was identified in the Exo screen (Feng et al., 2003) and shown to affect trafficking from the Golgi to the PM (Nieland et al., 2004). Although the protein target of this group is unknown, it is likely that sulfonamides exert their action indirectly by suppressing the vacuolar H^+-ATPase (V-ATPase) function, or directly as proton ionophores (Nieland et al., 2004). Chemical screens for inhibitors of endocytosis of toxins (shiga, cholera, and ricin) in mammalian cells facilitated the identification of compounds specifically affecting the retrograde toxin trafficking at the level of recycling endosomes and the Golgi (compound 75 and 134) (Saenz et al., 2007) or the EEs and TGN (Retro-1 and Retro-2) (Stechmann et al., 2010) level.

Receptor-mediated endocytosis in mammals has been tackled with the use of Ferristatin, which is a selective inhibitor of Tf-mediated iron uptake (Brown et al., 2004; Horonchik and Wessling-Resnick, 2008). Interestingly, Ferristatin has led to the discovery of an endocytic route for the PM-localized Tf receptor by inducing its enhanced internalization in a clathrin-independent manner (Horonchik and Wessling-Resnick, 2008). In plants, the limonoid Endosidin 1 (ES1) has been found to induce selective intracellular accumulation of some but not all PM cargoes (Robert et al., 2008). ES1 specifically blocks the trafficking of PM proteins at the level of TGN/EEs. Another compound from the Endosidin series, named ES5, has recently been implicated in the inhibition of PM protein recycling in both plant and mammalian cells (Drakakaki et al., 2011).

Chemical Effectors of Vacuolar Trafficking and Autophagy

Studies of vacuolar transport and biogenesis have been approached, for the first time, by forward chemical screens in yeast because of the conserved trafficking machinery between yeasts and plants (Zouhar et al., 2004; Figure 2; Table 1). Three compounds, called Sortin1, Sortin2, and Sortin3, induced secretion of the vacuolar protein carboxypeptidase Y in yeast and affected the morphology of *Arabidopsis* or yeast vacuoles (Zouhar et al., 2004). Although the targets of Sortins are not identified, evidence has been provided that Sortin1 targets the crosstalk between vacuolar biogenesis and transport (Rosado et al., 2011), whereas Sortin2 and Sortin3 affect vacuolar trafficking, possibly by inhibition of the endosomal trafficking (Chanda et al., 2009; Norambuena et al., 2008). Another small molecule linked to vacuolar trafficking is gravacin, which strongly inhibits gravitropism in *Arabidopsis* and blocks the trafficking of tonoplast markers to the vacuolar membrane by a yet unknown mechanism (Surpin et al., 2005).

Larger chemical genetic screens in plants have revealed several more small molecules affecting PM cargo trafficking to the vacuole or vacuolar morphogenesis, the targets of which remain to be identified (Drakakaki et al., 2011). For example, apart from perturbing the recycling pathway, ES5 has been proven to severely enhance the vacuolar accumulation of PM proteins (Drakakaki et al., 2011). Another compound derived from the same screen, namely C834, has been instrumental in identifying two distinct transport routes to the vacuole used by de novo-synthesized tonoplast intrinsic proteins (Rivera-Serrano et al., 2012).

A compound derived from the Exo screen (Feng et al., 2003), named Vacuolin-1, has been shown to induce the formation of large swollen vacuoles in different mammalian cell types through fusions of endosomes and lysosomes (Cerny et al., 2004).

In mammals and yeast, extensive research has gone into identifying small-molecule effectors of autophagy. One of the best examples of small molecules affecting autophagy is rapamycin, the well-known inhibitor of the phosphatidylinositol kinase target of rapamycin (TOR) (Noda and Ohsumi, 1998). Screens for chemical modifiers of rapamycin identified several small-molecule inhibitors and small-molecule enhancers of rapamycin (SMER) with an effect on autophagy in yeast and mammalian systems (Sarkar et al., 2007). Despite previous reports (Mahfouz et al., 2006), plants are sensitive to rapamycin (Xiong and Sheen, 2012); however, in contrast to mammals and yeasts, no direct link between rapamycin and autophagy has yet been shown in plants.

Special Considerations when Analyzing Drug-Induced Endomembrane Trafficking Phenotypes

Growing evidence suggests that, when targets of endomembrane effectors are not known, the interpretation of the observed phenotypes should account for possible effects of the compounds on the cytoskeleton dynamics. The endosomal maturation in mammalian cells is dependent on the microtubule network (Hehnly and Stamnes, 2007), as disruption of microtubules leads to dispersal of LEs and lysosomes throughout the cytoplasm (Huotari and Helenius, 2011). In turn, formation and motility of EEs and concurrent sorting of internalized proteins for either recycling or lysosomal degradation are largely dependent on the actin dynamics (Boulant et al., 2011; Ohashi et al., 2011). Similarly, stabilizing actin in plant cells by using chemicals interferes with endocytosis and vesicle transport (Dhonukshe et al., 2008). Examples exist where the observed endomembrane trafficking phenotype in the presence of a bioactive compound is not due to disturbance of the function of an endomembrane protein but rather is a result of a modulation of the cytoskeleton organization. This is illustrated by the endocytic inhibitor ES1, also called prieurianin (Robert et al., 2008; Tóth et al., 2012). Recent findings suggest that ES1 acts primarily as an actin stabilizer by an unknown mechanism without affecting the microtubules, thus possibly inhibiting intracellular vesicle movement. The effect of ES1 on actin dynamics has been observed in both plants and mammals, pointing to conservation of its molecular target in eukaryotes (Tóth et al., 2012). Another example concerns the use of Secramine, which affects the cellular functions of the Rho GTPase Cdc42 and, in this way, impairs the Golgi-to-PM vesicle trafficking but also inhibits actin polymerization (Pelish et al., 2006). Taken together, these examples corroborate the need to routinely test chemical compounds for direct effect on the cytoskeleton. Another consideration in studying endomembrane trafficking through chemical biology is the necessity to discriminate between primary compound-specific effects and general changes in the cellular homeostasis. Because all steps of intracellular vesicle trafficking are ATP- or GTP-dependent, energy depletion induced by the compound

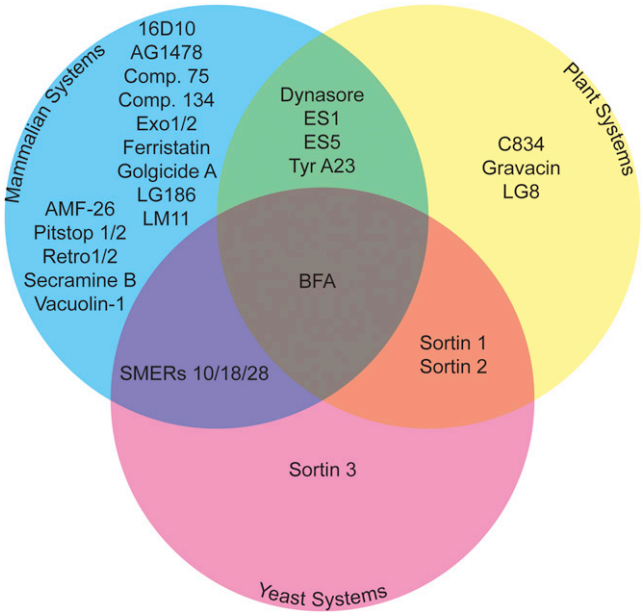


Figure 3. Distribution of Discussed Small Molecules with Activity in Mammalian, Yeast, and Plant Systems
Small molecules are distributed according to overall activity, thus not specific to endomembrane compartments.

hit would lead to a global disruption of the endomembrane system. Notably, cytotoxicity assays, such as those dealing with ATP content determination, could reveal the borderline dose and time of exposure, beyond which the compound hit induces unspecific responses (Thorne et al., 2010).

Perspectives and Future Challenges

Small molecules are powerful tools in deciphering highly dynamic and essential cellular processes, such as endomembrane trafficking. However, the subsequent identification of the molecular targets that underlie the observed phenotype remains a major challenge. Unlike medical research, the rate of compound target discovery in plant-based chemical genetic studies is still limited (Tóth and van der Hoorn, 2010). Although forward genetic screens for compound resistance remain a favorite approach for target identification in plants, in most cases, only downstream components of the pathway in which the compound acts are identified (Hicks and Raikhel, 2012). Affinity chromatography is an alternative powerful tool to identify proteins that physically interact with the compound (Frei et al., 2012), which still remains to be demonstrated in plants. Various compound deconvolution strategies have recently been developed, mostly in the human drug discovery field (Cong et al., 2012; Ho et al., 2011). Recent progress includes advances in chemoinformatics, providing increasingly fast and reliable computational tools to predict potential targets for orphan bioactive ligands to identify off-targets responsible for side effects and to propose novel targets of existing drugs (Laggner et al., 2012; Lounkine et al., 2012). Although the use of computational approaches for activity profiling of compounds in plants is limited by the insufficient structural biology data (Kumari and van der Hoorn, 2011), one can apply existing in silico ligand profiling methods to available structural data of other eukaryotes and then translate the predicted model to plants. Proteomics tools can also provide insights on the effect of small molecules at the whole proteome level, as exemplified by BFA (Takác et al., 2011), or at the level of individual organellar proteomes, as reported in mammals and plants (Drakakaki et al., 2012; Steuble et al., 2010). Furthermore, small-molecule targets identification can also benefit from coupling chemical genomics with semiautomated image acquisition and image analysis, which will allow us to assess quantitative differences in the phenotype caused by individual compounds (Ung et al., 2012).

However, compounds might have more than one target. One example in plants is gravacin, a compound that appears to have an effect both on the ER-to-vacuole trafficking through an unknown mechanism and on gravitropic responses through modulating the activity of a PM transporter (Rojas-Pierce et al., 2007; Surpin et al., 2005). Even when a small molecule has been optimized to specifically inhibit a certain target, other off-target effects might still be observed. This is exemplified by Pitstop 2, which, apart from inhibiting clathrin-mediated endocytosis (von Kleist et al., 2011), also appears to affect clathrin-independent endocytosis via an unknown mechanism (Dutta et al., 2012). Hence, a deeper understanding of the action mechanisms of newly described compounds is required.

The discernible degree of conservation of a number of endomembrane trafficking components in eukaryotes (Dacks et al., 2008) evokes one's expectations for the existence of universal chemical moieties impacting common vesicle transport routes. Many classical endomembrane trafficking inhibitors commonly used in mammalian systems have been successfully introduced as valuable tools in plant cell research (Figures 2 and 3). In turn, the validation of the intracellular effects of plant-specific inhibitors in mammalian cell cultures, as shown for ES5 and ES1/prieurianin (Drakakaki et al., 2011; Tóth et al., 2012), opens novel opportunities for translating the knowledge obtained from plant-based screens to human drug discovery. Ultimately, it would be tempting to define a set of general principles for using information derived from large-scale chemical genetic screens to decipher the evolution of the endomembrane system in eukaryotes. However, the evolutionary aspect of small molecules' specificity is closely related to the mode of action of the compound and the conservation of the respective binding sites. Small molecules can affect conserved protein motifs and remain active in different species (exemplified by BFA). In contrast, trafficking regulators, such as Exo 2 (Feng et al., 2004; Spooner et al., 2008), that target specifically a group of proteins in one kingdom are not necessarily active toward evolutionary conserved proteins in other eukaryotes, possibly due to speciation events. It remains to be seen if natural products (e.g., BFA) are more evolutionary specific compared to small molecules from large combinatorial (synthetic) libraries. Thus, addressing the degree of evolutionary specificity of a compound would require a thorough characterization of the small molecule mode of action.

ACKNOWLEDGMENTS

We thank Annick Bleys for help with manuscript preparation. This work was supported by the Flemish Agency for Innovation by Science and Technology by postdoctoral (to K.M.) and predoctoral (to W.D.) fellowships.

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